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BRIEF REPORT

First isolation of enteroaggregative *Escherichia coli* O104:H4 from a diarrhea case in Argentina**Claudia C. Carbonari^{a,*}, Natalia Deza^a, Mario Flores^b, Alejandra Gasparini^c, Eduardo Manfredi^a, Marta Rivas^a**^a Servicio Fisiopatogenia, Departamento de Bacteriología, Instituto Nacional de Enfermedades Infecciosas - ANLIS "Dr. Carlos G. Malbrán", Buenos Aires, Argentina^b Laboratorio de la Dirección de Patologías Prevalentes y Epidemiología, Ministerio de Salud de la Provincia de Chubut, Rawson, Provincia de Chubut, Argentina^c Centro Periférico de Salud Ramón Carrillo, Hospital Zonal de Trelew, Trelew, Provincia de Chubut, Argentina

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KEYWORDSEnteroaggregative
Escherichia coli;
O104:H4 serotype;
Diarrhea**Abstract**

We describe the first isolation of an enteroaggregative *Escherichia coli* (EAEC) O104:H4 strain associated with an acute diarrhea case in Argentina. Two multiplex PCRs (mPCR) were performed as screening of genes mPCR1 (*eae*, *lt*, and *st*) and mPCR2 (*lpaH*, *aggR*, *stx*₁ and *stx*₂). A mPCR to detect the *rfb*_{O104}, *fliC*_{H4} and *terD* genes, and PCR assays for the detection of pCVD432 plasmid, *aaiC* and *lpf*_{O113} genes were included. Biochemical and antimicrobial susceptibility assays as well as serotyping were performed. The identified *E. coli* strain was susceptible to all antimicrobials tested and harbored the *aggR*, *aaiC*, pCVD432 plasmid, *lpf*_{O113}, *rfb*_{O104}, *fliC*_{H4} and *terD* genes. Although serotype EAEC O104:H4 rarely spreads and sporadic cases have been reported, global concern increased after the large-scale outbreak in Europe in 2011. The finding of EAEC O104:H4 reinforces the need for improved methodologies for the detection of all *E. coli* pathotypes.

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PALABRAS CLAVE

Escherichia coli
enteroagregativo;
Serotipo O104:H4;
Diarrea

Primer aislamiento de *Escherichia coli* enteroagregativo O104:H4 de un caso de diarrea en Argentina**Resumen**

Se describe el primer aislamiento de una cepa de *Escherichia coli* enteroagregativo (EAEC) O104:H4 de un caso de diarrea aguda en Argentina. Se realizaron dos PCR múltiples como tamizaje: mPCR1 para los genes *eae*, *lt* y *st*, y mPCR2 para los genes *lpaH*, *aggR*, *stx*₁ y *stx*₂. Se incluyó una mPCR para detectar los genes *rfb*_{O104}, *fliC*_{H4} y *terD*, además de PCR simples para los genes del plásmido pCVD432, *aaiC* y *lpf*_{O113}. Se realizaron ensayos bioquímicos, de sensibilidad a los antimicrobianos y de serotipificación. La cepa de *E. coli* identificada fue sensible a todos los antimicrobianos ensayados y presentó los genes *aggR*, *aaiC*, plásmido pCVD432, *lpf*_{O113}, *rfb*_{O104}, *fliC*_{H4} y *terD*. Si bien EAEC O104:H4 es un serotipo poco común, se han comunicado casos esporádicos, pero la preocupación global aumentó después del brote masivo ocurrido en Europa en 2011. El hallazgo de EAEC O104:H4 refuerza la necesidad de mejorar las metodologías para la detección de todos los patotipos de *E. coli* en Argentina.

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Diarrheagenic *Escherichia coli* (DEC) strains are a common cause of diarrhea in humans. DEC strains are classified into five pathotypes: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and Shiga toxin-producing *E. coli*⁸ (STEC).

EAEC was first described in 1987 during a prospective study of pediatric diarrhea in Santiago, Chile⁸. In general, EAEC is associated with acute and persistent diarrhea in children and adults from industrialized and developing countries, international travelers and immunocompromised patients¹⁴.

The reservoir is unknown, but EAEC strains were isolated from the feces of different animals such as calves, piglets and horses, and contaminated food has been described as a possible vehicle for strain dissemination. Cases associated with EAEC infection are reported to be sporadic, but some outbreaks have been described, although the sources of infection have rarely been identified in those episodes⁹.

Several EAEC virulence-related genes have been described, however, their role in the clinical outcome of infection has not been completely defined. This *E. coli* pathotype is characterized by aggregative adherence (AA) to HEp-2 cells in a characteristic "stacked brick" pattern. The AA phenotype is associated with the presence of a 60 MDa plasmid (pAA), and the expression of one out of four distinct aggregative adherence fimbriae⁹ (AAFI, AAFII, AAFIG, and AAFIV). These AAFs are transcriptionally regulated by an AraC/XylS family activator called AggR. Under the control of *aggR* is the anti-aggregation protein gene (*aap*), present on the pAA-plasmid, and encoding a protein called dispersin, which promotes dispersal of EAEC on the intestinal mucosa to establish new foci of infection. AggR is also required to express the Aat dispersin translocator and the chromosomal cluster called *aggR*-activated island (*aai*), encoding a type VI secretion system. Other putative virulence factors not regulated by *aggR* are the EAEC heat-stable toxin 1 (EAST-1, encoded by the *astA* gene) and a set of toxins, known as serine protease autotransporters of

Enterobacteriaceae (SPATEs), which include the cytotoxic plasmid-encoded toxin^{3,10} (Pet).

The pathogenesis of EAEC infection is not fully understood due to the heterogeneity of the strains. A three-stage model based on *in vitro* and animal data was proposed. Stage I involves initial adherence to the intestinal mucosa and the mucus layer. Stage II comprises enhanced mucus production, apparently leading to the deposit of a thick mucus-containing biofilm encrusted with EAEC. This may contribute to EAEC diarrheagenicity and, perhaps, to its ability to cause persistent colonization, diarrhea and nutrient malabsorption. Stage III, suggested from histopathologic and molecular evidence, includes the elaboration of toxins or inflammation, which result in damage to the mucosa and intestinal secretion⁹.

The clinical profile is characterized by watery, mucoid, secretory diarrhea with low-grade fever, with or without vomiting. However, up to one third of patients with diarrhea by EAEC had grossly bloody stools. While malnutrition predisposes to persistent diarrhea, asymptomatic colonization with EAEC might cause malnutrition because of the persistent mucosal damage, becoming a possible and dangerous cycle⁸.

A few years ago, global concern about EAEC increased when a large-scale outbreak associated with a new Shiga toxin-producing/enteroaggregative *E. coli* pathotype occurred in Europe in May-June 2011. A total of 881 hemolytic uremic syndrome cases, 32 deaths and 3141 cases of diarrhea were reported. The STEC/EAEC outbreak strain belonged to the O104:H4 serotype². Since then, all countries have increased surveillance of this serotype in all diarrhea cases.

The aim of the present study was to describe the first isolation of an EAEC O104:H4 strain associated with a diarrhea case in Argentina.

On January 2013, a 6-year-old girl presented with a 72-hour history of acute diarrhea at Trelew Zonal Hospital, in the Province of Chubut. At the local laboratory, *E. coli* was isolated from a stool sample, and the strain was then

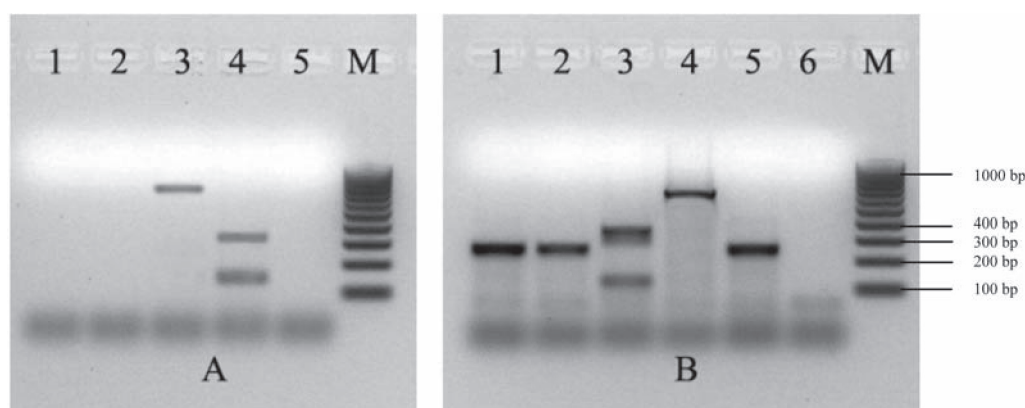


Figure 1 Multiplex PCRs (mPCR) used as screening of EPEC, ETEC, STEC, EIEC and EAEC. A. Multiplex PCR1 for the detection of *eae*, *lt*, and *st* genes. Lane 1: confluent culture of the diarrhea case; lane 2: isolated colony of the diarrhea case; lane 3: *E. coli* 2348/69 positive control for the *eae* gene (864 bp); lane 4: *E. coli* H10407 positive control for *lt/st* genes (322, 147 bp); line 5: reagent control; M: 100 kb molecular size marker. B. Multiplex PCR2 for the detection of *lpaH*, *aggR*, *stx*₁ and *stx*₂ genes. Lane 1: confluent culture of the diarrhea case; lane 2: isolated colony of the diarrhea case; lane 3: *E. coli* 110/05 positive control for *stx*_{1/stx}₂ genes (130, 346 bp); lane 4: *E. coli* C-481 positive control for the *lpaH* gene (619 bp); lane 5: *E. coli* 17-2 positive control for the *aggR* gene (254 bp); lane 6: reagent control; M: 100 kb molecular size marker.

sent to the National Reference Laboratory (NRL) for further characterization.

At the NRL, the confluent growth zone and colony pools were screened by two multiplex PCRs (mPCR). The mPCR1 enabled to characterize *eae* (EPEC), *lt*, and *st* (ETEC), and the mPCR2, *lpaH* (EIEC), *aggR* (EAEC), *stx*₁ and *stx*₂ (STEC) genes⁷. The mPCR for detection of *rfb*_{O104}, *fliC*_{H4}, and *terD* genes was carried out as described², with minor modifications. By single PCR assays, the sequence of the pCVD432 EAEC plasmid, the putative virulence genes, *aaiC*, and the long polar fimbria *lpf*_{O113}, were studied^{1,3,5}. The strain was characterized by biochemical tests⁶, serotyping¹¹, and antimicrobial susceptibility (nalidixic acid, amikacin, ampicillin, ciprofloxacin, chloramphenicol, streptomycin, gentamicin, tetracycline, trimethoprim-sulfamethoxazole and nitrofurantoin), based on 2011 CLSI guidelines⁴.

The *aggR* gene was detected by mPCR1 (Fig. 1). Serotyping and mPCR confirmed that the strain belonged to the O104:H4 serotype and was *terD*-positive (Fig. 2). The strain harbored the pCVD432 sequence, *aaiC* and *lpf*_{O113} genes. It was identified as *E. coli* by biochemical tests and was susceptible to all antibiotics tested.

A comparison of the virulence attributes between the Argentine O104:H4 strain and the German outbreak strain is shown in Table 1.

In the present study, the isolation of an EAEC O104:H4 strain from a diarrhea case was described for the first time in our country.

In Argentina, few data are available on the frequency of EAEC strains in diarrhea cases. In a study performed by Ortiz *et al.* in Mendoza in 1998, *E. coli* strains isolated from 140 children under 24 months of age, suffering from acute (n=120), persistent (n=16) and chronic (n=4) diarrhea were studied by the HEp-2 cell-adherence assay. The aggregative adherence (AA) pattern was recognized in strains from 28 (20%) cases. In another study reported by Quiroga *et al.*, the onset time of the first asymptomatic infection caused by the different DEC categories was studied in 44 children

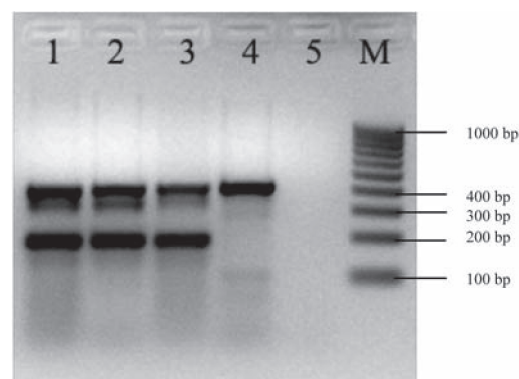


Figure 2 Multiplex PCR for detection of *rfb*_{O104}, *fliC*_{H4}, and *terD* genes. Multiplex PCR for the detection of *rfb*_{O104}, *fliC*_{H4}, and *terD* genes. Lane 1: confluent culture of the diarrhea case; lane 2: isolated colony of the diarrhea case; lane 3: *E. coli* 870 positive control for the *terD* gene (434 bp), *rfb*_{O104} gene (351 bp) and *fliC*_{H4} gene (201 bp); *E. coli* FP595/11 lane 4 positive control for the *terD* gene (434 bp); lane 5: reagent control; M: 100 kb molecular size marker.

since birth until the first 20 months of age in 2000. In all children, at least one DEC strain was detected, and a total of 510 (33.5%) DEC strains were isolated from 1524 fecal samples, with EAEC being the most frequent (31.4%) pathotype identified. Rüttler *et al.* published the characterization of 87 *E. coli* strains isolated from patients under 2 years of age, suffering from acute diarrhea in Mendoza in 2002, using the reference HEp-2 assay and AAF/I- and EAST1-PCR assays. Based on the adherence to HEp-2 cells, 22 (25%) strains showed the localized (LA) pattern, 18 (21%) the aggregative (AA) pattern, and 10 (11%) the diffuse (DA) pattern. Twenty-five strains were positive by EAST1-PCR, whereas only 13 of them expressed the AA pattern on HEp-2 cell-adherence assay, and 13 strains were positive by AAF/I-PCR but only 8 showed the AA

Table 1 Comparison between the Argentine and German *E. coli* O104:H4 strains

Gene/Target	Target description	Argentine <i>E. coli</i> O104:H4 strain	German <i>E. coli</i> O104:H4 strain
<i>aggR</i>	Transcriptional activator	+	+
<i>aaiC</i>	AaiC, secreted protein	+	+
pCVD432	pCVD432 plasmid sequence	+	+
<i>lpf</i> _{O113}	Structural subunit of long polar fimbriae of STEC O113	+	+
<i>st-b</i>	B subunit heat-stable enterotoxin	–	–
<i>lt-A</i>	A subunit heat-labile enterotoxin	–	–
<i>ipaH</i>	Invasion plasmid antigen H	–	–
<i>ehxA</i>	Hemolysin	–	–
<i>eae</i>	Intimin	–	–
<i>stx</i> ₁	Shiga toxin 1	–	–
<i>stx</i> ₂	Shiga toxin 2	–	+
<i>rfb</i> _{O104}	Region of the O104 somatic antigen	+	+
<i>fliC</i> _{H4}	H4 flagellar antigen	+	+
<i>terD</i>	Tellurite resistance	+	+

pattern. More results were reported in 2010 from one study carried out in Corrientes by Medina *et al.*, where 120 children aged 1 month to 14 years with acute diarrhea were cared for in health centers in poor neighborhoods of the city. All children had urban housing with drinking water supply and toilet. DEC strains were detected in 41 (37%) samples, and EAEC strains in 17.1% of the cases¹².

The EAEC O104:H4 strain described in the present study harbored the *aggR*, *aaiC*, pCVD432 plasmid, *lpf*_{O113}, *rfb*_{O104}, *fliC*_{H4} and *terD* genes, as the STEC/EAEC isolated during the outbreak in Europe, however, it was negative for the *stx*₂ gene as is shown in Table 1.

The Argentine strain was susceptible to all the antimicrobial agents tested, while the O104:H4 isolated in Germany showed an ESBL phenotype (resistant to all penicillins and cephalosporins and susceptible to carbapenems), a CTX-M-15 genotype² and, was also resistant to co-trimoxazole (trimethoprim-sulfamethoxazole) and susceptible to fluoroquinolones (ciprofloxacin) and aminoglycosides (gentamicin, tobramycin).

Serotype EAEC O104:H4 is not commonly spread. Sporadic cases were reported before the 2011 outbreak. Two isolates from patients with HUS in Germany in 2001, one in France in 2004, one from a HUS case in Korea in 2004, two from HUS cases in the Republic of Georgia in 2009, and one from a diarrhea case in Finland in 2010 were reported. The isolates from Germany, Finland and the Republic of Georgia were EAEC/STEC¹³. In a study of childhood diarrhea in Mali, EAEC O104:H4 was isolated from three children with moderate to severe diarrhea³.

The finding of an EAEC strain of serotype O104:H4 reinforces the need to implement methodologies for the detection of all *E. coli* pathotypes. Although O104:H4 is an uncommon serotype, the 2011 European outbreak caused great damage to public health, marking a turning point in

terms of surveillance. For this reason, it is important to emphasize that the discovery of new pathotypes is possible, due to the ability of *E. coli* strains to horizontally acquire specific genetic elements known as pathogenicity islands (PAI), and *stx* genes from free bacteriophages in the environment and in mammalian hosts; with an improved adaptation of the bacteria to human hosts. Due to the behavior of this genome, the Reference Laboratories must be able to detect and characterize the emerging new pathogens.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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